APPENDIX A

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Lack of Promiscuity in Autoantigen-Specific H and L Chain Combinations as Revealed by Human H and L Chain "Roulette"[1]

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ABSTRACT. Individual H or L chains from a human autoantibody were used to search for other L or H chains that could form antigen-binding fragments, Fab, with the same specificity. The parent Fab (SP1.2) exhibits high affinity binding for thyroid peroxidase (TPO), a 107-kDa protein that is the major autoantigen in human autoimmune thyroiditis. This autoantibody "roulette," performed by using Ig H and L chain gene libraries expressed in bacteria, increased the frequency of TPO-binding clones in the new libraries. However, the frequency was still much lower than would be the case if promiscuous combinations with a variety of H or L chains were compatible with specific Ag binding. Nucleotide sequence analysis of the H and L chains of the new TPO-binding clones revealed even more restriction. Thus, with the SP1.2 H chain, all 11 new Fab utilized L chains from the same $V_\kappa 1$ family germline gene as SP1.2 itself. Similarly, five of six H chains "captured" by the SP1.2 L chain were very closely related to the SP1.2 H chain. However, one totally different H chain was isolated: SP4.6 has a V_H region that differs substantially from that of SP1.2. SP4.6 also has a distinct D region, uses a different J_H, and, unlike SP1.2, which is an IgG1, belongs to subclass IgG4. The affinities for TPO of SP4.6 (with the different H chain) and SP1.20 (which had the least mutated L chain germline gene) were similar to that of SP1.2 ($\sim 10^{-10}$ M). As expected, the SP1.2 and SP1.20 Fab, which have the same H chain and closely related L chains, bound to the same domain on TPO. However, a similar domain on TPO was recognized by both SP4.6 and SP1.2, despite the fact that their V, D, and J regions are quite different. This observation raises the possibility that the L chain is critical in defining epitope specificity, even in the presence of completely different D regions and nonidentical V_H regions. *Journal of Immunology,* 1993, 150: 880.

A hallmark of autoimmune thyroid destruction in humans is the presence in serum of high affinity IgG class autoantibodies to TPO,[3] the primary enzyme involved in thyroid hormone synthesis (reviewed in Reference 1). TPO is a glycoprotein of~ 107 kDa expressed on the surface of thyroid cells (reviewed in Reference 1). Human autoantibodies to TPO are not monoclonal, as evidenced by the contribution of different IgG subclasses and L chain types in the same patient (2, 3).

We have cloned three human IgG1/κ autoantibodies (SP1.2, SP1.4, and SP1.5; previously called SP2, -4, and -5) that bind TPO specifically and with high affinity (4, 5). These autoantibodies were obtained by expressing random combinations of H and L chain Ig genes as Ag-binding fragments, Fab, in a bacteriophage λ library (6) (Fig. 1*A*). The cDNA in this library was prepared from B cells infiltrating the thyroid gland of a patient with autoimmune

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[3] Abbreviation used in this paper: TPO, thyroid peroxidase.

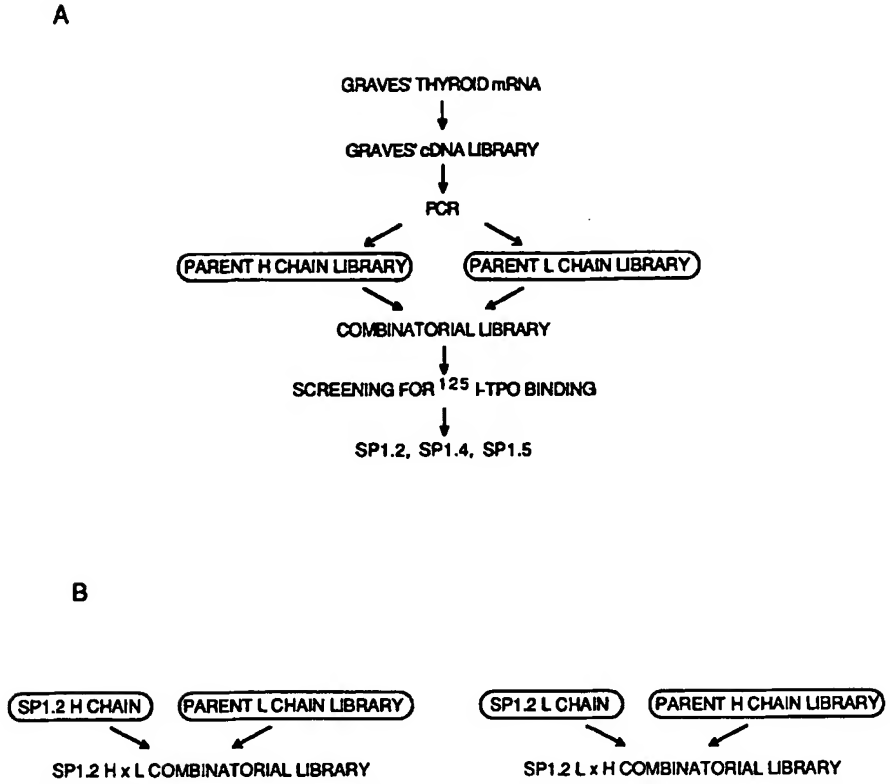

FIGURE 1. *A*, Outline of the approach (6) used to construct parent H and L chain libraries and the combinatorial library from which TPO-specific Fab SP1.2, SP1.4, and SP1.5 were obtained (4, 5). *B*, combinatorial libraries constructed by using SP1.2 H (or L) chain with the parent L (or H) chain. *PCR*, polymerase chain reaction.

thyroid disease. The three TPO autoantibody Fab share the identical Ig H chain. The L chains are not the same but are derived from the same V_κ germline gene, HSIGKLO12 (7).

Because of the random nature of the H and L chain combinations in our cDNA library, the question arose as to whether the SP Fab autoantibody H chain (or L chain) could combine with a variety of other L chains (or H chains) in the parent library and still bind to TPO. Therefore, we used the SP1.2 H (or L chain) to search for other L (or H) chains in the parent libraries that could form a Fab capable of binding TPO (Fig. 1*B*). Recently, following a similar approach, it has been reported that there is notable promiscuity in the H and L chain combinations of murine Fab that bind the hapten nitrophenyl phosphonamidate (8). In contrast, our "roulette" experiments demonstrate more restriction in the H and L chain combinations for high affinity binding to a human autoantigen, TPO.

Materials and Methods

Library construction and screening

The plasmid of clone SP1.2 (4) was digested with *Xho*I and *Spe*I to release the H chain cDNA insert. Similarly, the L chain cDNA fragment was obtained by *Xba*I and *Sac*I digestion. The inserts were gel purified and ligated into Immunozap H and L arms (Stratacyte, La Jolla, CA), respectively, to generate a clonal SP1.2 H chain library and a clonal SP1.2 L chain library, respectively (Fig. 1*B*). These libraries were amplified and the DNA was extracted as described (4). The DNA from the SP1.2 H chain library was digested with *Hind*III followed by *Eco*RI and was ligated with the DNA prepared from the original L chain library (4). Similarly, the DNA from the SP1.2 L chain library was digested with *Mlu*I followed by *Eco*RI and was ligated with the DNA from the original H chain library (4). The original H and L libraries contained >80% inserts of the correct size. The combinatorial libraries were screened in XL1-Blue cells by conventional techniques (9), by using secreted human rTPO (10) that had been labeled with ^{125}I (to a specific activity of 10 to 20 μCi/μg of protein) by the Iodogen method (11). TPO-binding plaques were cloned to homogeneity and plasmids were excised from the Immunozap bacteriophage by using the helper phage R408, according to the Stratacyte protocol. Nucleotide sequencing of the cDNA inserts was performed by the dideoxynucleotide chain termination method (12).

Fab expression

Fab were expressed as soluble proteins in XL1-Blue cells, as described previously (5). In brief, protein synthesis was induced with 1 mM isopropylthiogalactopyranoside (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C. The

Table I
Frequencies of ^{125}I-TPO-binding clones in roulette of SP1.2 H and L chains

Fab Combinatorial Library	Frequency	Plaques Screened
SP H chain × SP L chain	1:60,000	180,000
SP1.2 L chain × SP H chain	1:5,000	30,000
SP1.2 H chain × SP L chain	1:500	15,000

cells were then pelleted, frozen at −20°C, and resuspended in 0.02 volumes of 10 mM Tris, pH 8.0, containing 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.1 mM PMSF (all from Sigma). The suspension was sonicated, membranes were pelleted by centrifugation at 4000 × *g*, and the Fab were affinity purified from the supernatant by using a protein G-Sepharose column (Pharmacia, Piscataway, NJ).

Fab binding of ^{125}I-TPO

As described previously (5), Fab diluted in assay buffer (0.15 M NaCl containing 10 mM Tris·HCl, pH 7.5, and 0.5% BSA) were incubated with ^{125}I-TPO (~25,000 cpm) and mouse mAb to human κ L chains (QE11; Recognition Sciences, Birmingham, UK), in a total volume of 200 μl. After 1 h at room temperature, 100 μl of Sac-cel (donkey anti-mouse Ig covalently coupled to cellulose; IDS, Boldon, Tyne, and Wear, UK) were added, and the incubation was continued for 30 min. After addition of 1 ml of assay buffer and vortexing, the mixture was centrifuged for 5 min at 1000 × *g* to sediment the immune complexes, which were then counted to determine the percentage of radiolabeled TPO bound. The affinities of the Fab for TPO were determined by Scatchard analysis (13) from values obtained in the presence of increasing concentrations of unlabeled TPO. The data presented are the mean ± SEM of triplicate determinations.

Competition between Fab for binding to TPO

One Fab was immobilized by incubation (total volume, 200 μl) with murine mAb anti-human κ (QE11) for 1 h at room temperature. After incubation with 100 μl of Sac-cel

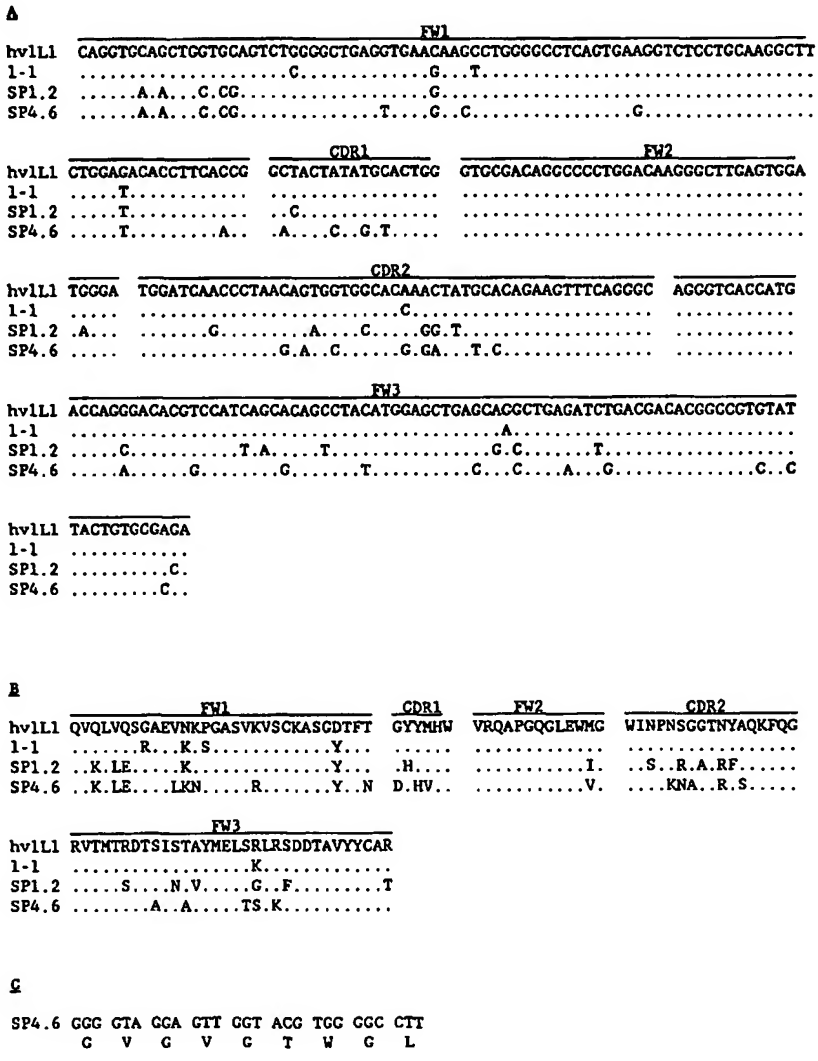

FIGURE 2. *A*, Nucleotide sequences of the V_H region of TPO-specific Fab SP1.2 and SP4.6, compared with germline genes hv1L1 (16) and 1–1 (14). *B*, derived amino acid sequence (in single-letter code) of the V_H regions of SP1.2 and SP4.6, again in comparison with hv1L1 and 1–1. In *A* and *B*, *dots* represent identical nucleotides or amino acids, respectively. CDR and framework regions (*FW*) are indicated according to Kabat et al. (26). *C*, nucleotide and derived amino acid sequences of the SP4.6 D region. These sequence data are available from GenBank (accession number M82813 for SP1.2) and from EMBL (accession number Z15084 for SP4.6).

(30 min at room temperature), the complexes were diluted in assay buffer (see above) and centrifuged at 1000 × g (5 min at 4°C). The pellets were resuspended in normal human serum diluted 1/30 in assay buffer, to saturate remaining anti-κ binding sites. In a separate set of tubes, increasing concentrations of "free" Fab were preincubated with ^{125}I-TPO for 1 h at room temperature. Aliquots (100 μl) were then incubated for 30 min with the immobilized Fab pellets and washed with assay buffer, and radioactivity bound to the Sac-cel was counted. Nonspecific binding (~2% of total counts added) was substracted to provide values for specific binding to TPO.

Results

Frequencies of ^{125}I-TPO-binding clones

Combining the L chain of SP1.2 with the SP parent H chain library yielded a combinatorial library of ~10^7 PFU (SP1.2 L × H library). Similarly, the combination of the SP1.2 H chain with the SP parent L chain library resulted in a library of ~10^7 PFU (SP1.2 H × L). As expected, this "roulette" with either the SP1.2 L or H chain led to a greater frequency of TPO-binding clones than was detected in the original screening of the parent library (Table I). Further, the frequency of TPO-binding clones was 10-fold greater in the SP1.2 H × L library than in the SP1.2 L × H library.

TPO-binding clones from the SP1.2 L × H library

We originally reported (4) that the V_H of SP1.2 appears to be derived from the V_H1 family germline gene 1-1 (14). V_H1-1 was first reported to be a pseudogene, but other investigators have suggested that 1-1 is a functional gene (15). More recently, another V_H1 germline gene, hv1L1, has been described (16) that is closely related to 1-1. At the nucleotide level, SP1.2 is more homologous to hv1L1 (93%) than to 1-1 (90%) (Fig. 2*A*).

Determination of the nucleotide sequences of the six new TPO-binding clones isolated from the SP1.2 L × H library revealed that the VDJ regions of five (SP1.7 to -11) are almost identical to those of the original SP1.2 (Table II). In the V_H region, three clones (SP1.9, SP1.10, and SP1.11) are identical to SP1.2 H chain and two clones (SP1.7 and SP1.8) differ from the original SP1.2 H chain by only one amino acid (proline and leucine for Ala-24, respectively). The D regions of SP1.7 to -11 are identical and differ from the SP1.2 D region by a single silent base substitution. The J regions of SP1.7 to -11 are of the J_H6 family (15). Re-examination of the SP1.2 DJ regions indicates that this clone, too, is a J_H6 (rather than a J_H3) combined with a very short D region.

One of the six new clones (SP4.6) from the SP1.2 L × H library differs more substantially from SP1.2 as well as from SP1.7 to -11 (Table II). Both SP4.6 and SP1.2 are members of the V_H1 family and may be derived from the germline gene hv1L1 (16) (Fig. 2*A*). However, there are major differences (24 amino acids) in the V_H regions of SP4.6 and SP1.2 (Fig. 2*B*). In addition, the SP4.6 D region (Fig. 2*C*) is quite different from that of SP1.2 and does not resemble any published D region sequence. Further, the SP4.6 J region is a J_H4 truncated at its 5′ end. The most surprising finding was that the hinge region sequence indicates that SP4.6 is an IgG4, in contrast to SP1.2 and SP1.7 to -11, which are IgG1. The C region primer used for the polymerase chain reaction in constructing the SP H chain parent library (CH1; Stratacyte, San Diego CA) is described as an IgG1 primer with the capacity to cross-prime with other IgG subclasses.

TABLE II
Characteristics at the nucleotide level of 17 TPO-binding clones in roulette of SP1.2 H and L chains[a]

SP1.2 L × H (6 clones)	V_H (%)	D	J_H	Subclass
SP1.7; -1.8	94	SP1.2	6	IgG1
SP1.9; -1.10; -1.11	94[b]	SP1.2	6	IgG1
SP4.6	90	Non-SP1.2	4	IgG4
SP1.2 H × L (11 clones)	**V_K (%)**		**J_K**	
SP1.17; -1.19	92[c]		2	
SP1.14; -1.15; -1.22	93[d]		2	
SP1.21	93[d]		?	
SP1.12	94		1	
SP1.13	93		2	
SP1.16	92		2	
SP1.18	93		2	
SP1.20	95		1	

[a] Homology of V_H and V_L genes is calculated for putative germline genes hv1L1 and HSIGKL012, respectively. The D regions are described as resembling SP1.2 (4) or not resembling SP1.2 (Fig. 2*C*).
[b] 100% homologous with SP1.2 H.
[c] 100% homologous with SP1.2 L.
[d] Identity of V_K for SP1.14, SP1.15, SP1.22, and SP1.21.

TPO-binding clones from the SP1.2 H × L library

The nucleotide sequences of the L chains were determined in 11 TPO-binding clones in the SP1.2 H × L library (Table II; Fig. 3). At the amino acid level, the $V_κ$ regions of nine clones are very similar to, and two clones (SP1.17 and SP1.19) are identical to, the original SP1.2 L chain (Fig. 4*A*). Four of the nine clones (SP1.14, SP1.15, SP1.21, and SP1.22) have identical $V_κ$ regions. All 11 new L chain clones appear to be derived from the $V_κ$ germline gene HSIGKLO12 (7). This gene is almost identical to HUMIGKLVJ (17), with which we originally compared SP1.2 (4). SP1.20 is the closest (95%) to the putative germline gene (Table II). The $J_κ$ regions of 10 of the 11 new clones belong to $J_κ$1 or $J_κ$2 (Fig. 4*B*). We cannot assign the $J_κ$ region of SP1.21. SP1.14, SP1.15, and SP1.22 have identical $V_κ$ and $J_κ$ sequences. Because clones SP1.17 and SP1.19 have $J_κ$2 regions (like SP1.2) these clones are, overall, identical to SP1.2.

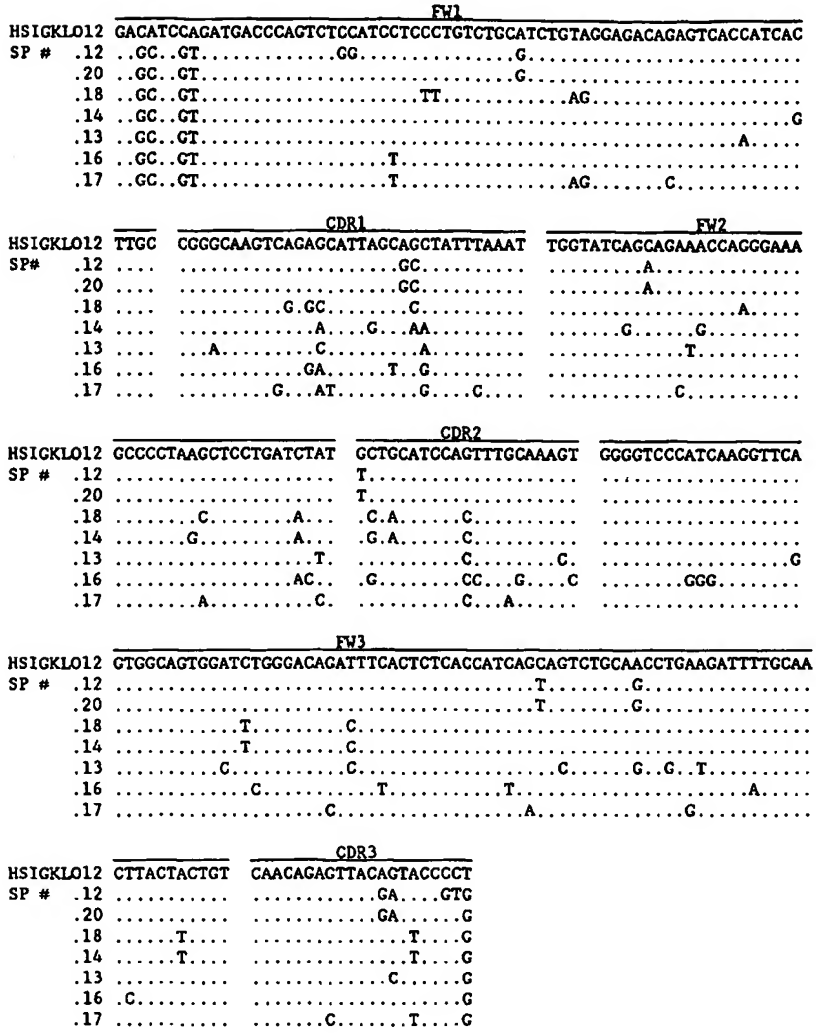

FIGURE 3. Nucleotide sequences of the V_κ regions of TPO-specific Fab SP1.12 through SP1.22, compared with the closest germline gene, HSIGKLO12 (7). The sequence of clone SP1.14 is identical to those of SP1.15, SP1.21, and SP1.22. Clone SP1.17 is identical to SP1.19 and both have the same sequence as SP1.2. *Dots*, identical nucleotides. CDR and framework regions (*FW*) are indicated according to Kabat et al. (26). These sequence data are available from EMBL under accession numbers Z15073 to Z15083 (SP1.12 to SP1.22).

Affinities for TPO of selected Fab

We expressed and purified the proteins for two of the newly isolated Fab clones, SP4.6 and SP1.20. SP4.6 was selected because of its distinctive H chain and SP1.20 because its L chain was the least mutated from the putative germline gene. Purified SP1.2 was already available (5). The affinities for TPO of the SP1.20 and SP4.6 Fab, calculated by Scatchard analysis (13) (Fig. 5), are very similar to each other (K_d, $1.0 \pm 0.2 \times 10^{-10}$ M and $1.4 \pm 0.3 \times 10^{-10}$ M, respectively) and to that of SP1.2 ($0.8 \pm 0.1 \times 10^{-10}$ M) (means ± SEM).

Binding domains on TPO for SP Fab

To determine whether there was overlap in the domains on TPO recognized by SP4.6, SP1.20, and SP1.2, we performed studies with an immobilized SP1.2 Fab. As expected, preincubation of ^{125}I-TPO with increasing concentrations of free SP1.2 inhibited the subsequent binding of the Ag to the immobilized SP1.2 (Fig. 6). The SP4.6 and SP1.20 Fab were equally effective, indicating that the binding domains of all three Fab overlapped. No competition was observed with another anti-TPO Fab, TR1.9, cloned from another patient.[4]

Discussion

In the present study, we chose a single H or L chain already known to confer high affinity ($\sim 10^{-10}$ M) specific binding for a large (107-kDa) protein autoantigen, TPO. We used this SP1.2 H (or SP1.2 L) chain to search for other L (or H) chains that could form a Fab capable of binding TPO. That is, we "spun the wheel" of the H and L chain repertoire of activated B cells infiltrating the patient's thyroid gland. As expected, the frequency of TPO-binding clones in the libraries generated by this biased recombination (1:500 for SP1.2 H × parent L chain and 1:5000 for SP1.2 L × parent H chain) was higher than in the original random combinatorial library (1:60,000) (4).

[4] Chazenbalk, G. D., Portolano, S., Russo, D., Hutchinson, J. S., Rapoport, B., McLachlan, S. "Human organ-specific autoimmune disease: molecular cloning and expression of an autoantibody gene repertoire." *Submitted for publication.*

A

		FW1	CDR1	FW2	CDR2
HSIGKLO12		DIVMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS
SP #	.12	EL....G.....	.....A...	...K.....	S.....
	.20	EL.....	.....A...	...K.....	S.....
	.18	EL.....E.....	...RA..T...	.....R...N....	GT.T...
	.14	EL.....S.	....N.GK...	..R.....E....	GT.T...
	.13	EL.....	.T..T..R...	....I.....F	A.....T
	.16	EL.....	....D..R...	.....H	G..T.E.
	.17	EL.....E..T.....	...EN..R.S.	....Q.....S	...T...

		FW3	CDR3
HSIGKLO12		GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
SP #	.12	.....	....D..
	.20	.....	.....
	.18	.....F.....F.	.....S.
	.14	.....F.....F.	.....S.
	.13	.....G.....T...D.....	.....
	.16	...G.....Y.....	.....
	.17	.....H.....N....G.....	..T..S.

B

		CDR3	FW4
SP #	.12	GACACGTTC	GGCCACGGGACCAAGGTGGAAATCAAACGAACT
	.21	CCG..T...	....CT.....GA.....G.....
	.20	TGG.....	.....
	.18	T....T...	.....G.....C....G..AG.....
	.14	T....T...	.....G.....C....G..AG.....
	.13	T....T..T	.....G.....C....G.....G
	.16	TT...T..T	.....G.....C....G.....G.....G
	.17	TT...T..T	.....G.....C....G.....G

C

		CDR3	FW4	
SP #	.12	DTF	GHGTKVEIKRT	JK1
	.21	P..	.P..R..V...	JK?
	.20	W..	.....	JK1
	.18	Y..	.Q...L..E..	JK2
	.14	Y..	.Q...L..E..	JK2
	.13	Y..	.Q...L.....	JK2
	.16	F..	.Q...L.....	JK2
	.17	F..	.Q...L.....	JK2

FIGURE 4. *A*, Derived amino acid sequences of V_κ regions of anti-TPO Fab SP1.12 through SP1.22, compared with the closest germline gene, HSIGKLO12 (7). *B* and *C*, nucleotide (*B*) and derived amino acid (*C*) sequences of the J_κ regions of SP1.12 through SP1.22. *Dots*, identical nucleotides. CDR are indicated according to Kabat et al. (26).

However, the frequency was still lower (Table I) than would be the case if promiscuous binding to a variety of H or L chains was compatible with specific Ag binding.

The antibody repertoire in thyroid tissue B cells of patients with autoimmune thyroid disease is relevant to this discussion. This tissue is enriched, compared with draining lymph nodes and peripheral blood, in B cells actively secreting autoantibodies to the three major thyroid autoantigens, i.e., TPO, thyroglobulin, and the thyrotropin receptor (reviewed in Reference 18). This bias makes even more remarkable the paucity of H and L chains from this patient that are capable of combining with the preselected L or H chain to form a functional TPO binding site. For example, frequencies of 1:50 for a functional H chain have been reported in libraries from a mouse immunized with influenza hemagglutinin or in a human immunized with tetanus toxoid (8).

Nucleotide sequence analysis of the H and L chains of the new TPO-binding clones reveals even more restriction. Thus, with the SP1.2 H chain, all 11 new clones utilized L chains from the same $V_\kappa 1$ family germline gene. This germline gene was also used by the three other TPO Fab already described (4, 5). Amino acid substitutions predominate in the CDR regions. Overall, taking into account the V_κ and J_κ regions of the L chains of 11 new clones and the previously described SP1.2, SP1.4, and SP1.5 (5), only 10 were distinct, because several were identical to the SP1.2 L chain or to each other.

Additional evidence for restriction was the very limited variety of H chains "captured" by the SP1.2 L chain in generating new TPO-binding clones. Five of the six clones were very closely related to the SP1.2 H chain, which is also shared by the previously identified SP1.4 and SP1.5 clones (5). However, unlike the L chains, we did isolate one H chain, SP4.6, that differs from all others in the following respects: (i) there are 24 amino acid differences between the V_H regions of SP4.6 and SP1.2, although both may be derived from the same $V_H 1$ germline gene, hvlLl; (ii) the D region is totally distinct; (iii) SP4.6 uses a different J region; and (iv) SP4.6 belongs to the IgG4 subclass. The SP4.6 H chain is more rare in the parent

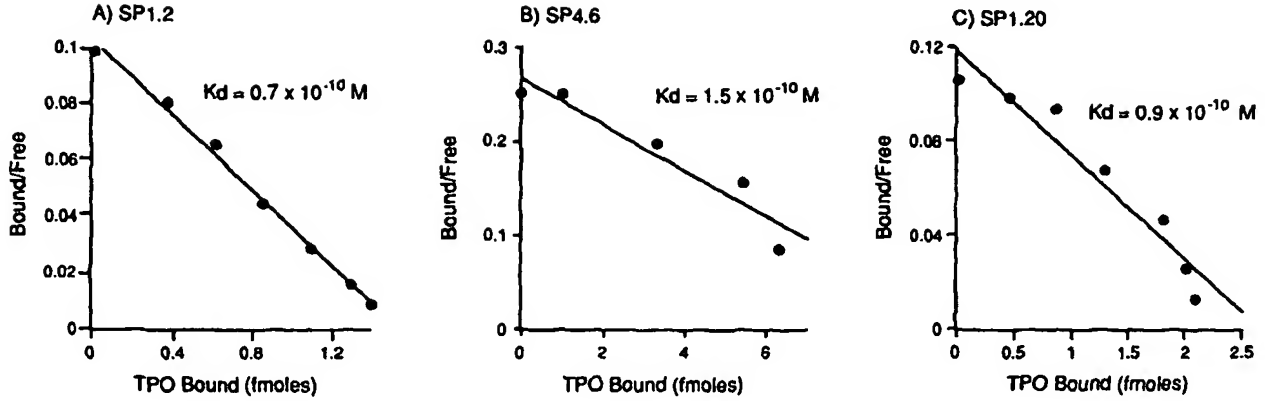

FIGURE 5. Scatchard analysis (see *Materials and Methods*) of the affinities of SP1.2, SP4.6, and SP1.20 for TPO. One of three representative experiments is shown for each Fab.

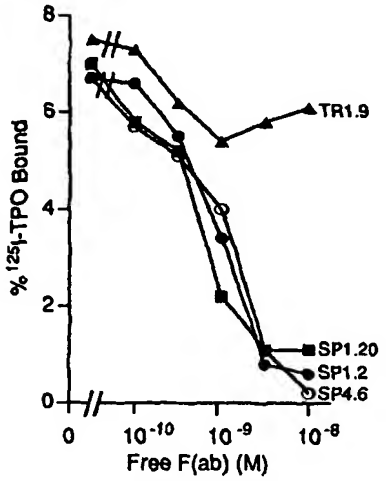

FIGURE 6. Binding domains on TPO for the SP1.2, SP4.6, and SP1.20 Fab. ^{125}I-TPO was preincubated in the absence or presence of increasing concentrations of SP4.6, SP1.20, and SP1.2 (*Free F(ab)*). Fab TR1.9 was obtained from another patient.[4] The ability of these complexes to bind to immobilized SP1.2 was then determined. The results are expressed as percentage of ^{125}I-TPO bound after subtraction of background values (~2%) obtained by using buffer alone.

library than is SP1.2 and related H chains, possibly because the oligonucleotide primer used to generate this library is suboptimal for IgG4. It is likely that the SP4.6 H chain was only found because of the bias introduced by the roulette approach.

In addition to providing data on the frequency and characterization of TPO-binding H and L chain combinations, our study illustrates important features with respect to antibody affinity and binding domains. These TPO autoantibodies have affinities for Ag several orders of magnitude greater than those reported for naturally occuring, polyreactive autoantibodies (K_d, 10^{-3} to 10^{-7} M) (19). It is possible that the screening approach that we used permits identification only of clones capable of high affinity TPO binding. Clones with lower affinity for TPO may, in the future, be identified more easily by using the phage display technique (20). However, this is not our present goal because TPO autoantibodies in patients' sera have high affinities (21), similar to our recombinant Fab.

It is of interest that the affinity of SP1.20 is high (similar to that of SP1.2) even though its V_κ chain is less mutated than that of SP1.2 (95% and 81% homology, respectively, to the germline gene). Assuming that both genes are, indeed, derived from HSIGKLO12, it is possible that affinity maturation of the L chain does not play a critical role in determining the affinity of the Fab for TPO.

The most unexpected finding in our study, in our estimation, concerns the TPO-binding domains of the SP Fab. Thus, in accordance with the data of Radic et al. (22) concerning murine autoantibodies to DNA, it was anticipated that both the SP1.2 and SP1.20 Fab, which have the same H chain and have closely related L chains, would interact with overlapping epitopes and bind to the same domain on TPO. However, a similar domain on TPO was recognized by both SP4.6 and SP1.2, despite the fact that their V, D, and J regions are quite different. This finding contrasts with the data of Martin et al. (23), who found that polyspecific autoantibody activity could only be generated with a specific D region. Our data were also unexpected in view of the extensive analysis by Kabat and Wu (24) of V region H and L chain combinations, which suggested V_H dominance in defining antibody specificity in many instances. However, as also suggested by Kabat and Wu (24) for some antibodies and as demonstrated by Smith-Gill et al. (25) for some murine antibodies to lysozyme, our findings raise the possibility that the L chain is critical in defining epitope specificity, even in the presence of completely different D regions and nonidentical V_H regions. With respect to our clones, it is possible that molecular modeling of SP4.6 and SP1.2 would reveal regions of similarity in their three-dimensional conformations, despite the dissimilarities in their CDR, particularly CDR3.

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